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Lab Resource: Stem Cell Line

Generation of spinocerebellar ataxia type 3 patient-derived induced pluripotent stem cell line SCA3.A11

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ABSTRACT

Spinocerebellar ataxia type 3 (SCA3) is a dominantly inherited neurodegenerative disease caused by a CAG-repeat expanding mutation in *ATXN3*. We generated induced pluripotent stem cells (iPSCs) from a SCA3 patient by electroporation of dermal fibroblasts with episomal plasmids encoding *L-MYC*, *LIN28*, *SOX2*, *KLF4*, *OCT4* and short hairpin RNA targeting *P53*. The resulting iPSCs had normal karyotype, were free of genomically integrated episomal plasmids, expressed pluripotency markers, could differentiate into the three germ layers *in vitro* and retained the disease-causing *ATXN3* mutation. This iPSC line could be useful for the investigation of SCA3 disease mechanisms.

Resource table.

Name of Stem Cell line	SCA3.A11
Institution	University of Copenhagen
Person who created resource	Susanne K. Hansen
Contact person and email	Jørgen E. Nielsen, jnielsen@sund.ku.dk
Date archived/stock date	Feb 5, 2013
Origin	Human skin fibroblasts
Type of resource	Induced pluripotent stem cells derived from a patient with spinocerebellar ataxia type 3 (SCA3), CAG-repeat lengths: 23/83
Sub-type	Cell line
Key transcription factors	Episomal plasmids containing *SOX2*, *L-MYC*, *KLF4*, *LIN28*, *OCT4* and *shP53* (Okita et al., 2011)
Authentication	Identity and purity of the cell line were confirmed by karyotyping, integration analysis, pluripotency analysis and confirmation of the CAG-repeat expanding mutation in *ATXN3* (Fig. 1)
Link to related literature	http://www.nature.com/nature/journal/v480/n7378/full/nature10671.html (Koch et al., 2011)
Information in public databases	Not available
Ethics	The study was approved by the regional scientific ethical committee in the Capital Region of Denmark and informed consent was obtained from the patient (H-4-2011-157).

Resource details

Spinocerebellar ataxia type 3 (SCA3) is a dominantly inherited neurodegenerative disease caused by a mutation expanding the CAG-repeat of the *ATXN3* gene encoding ataxin-3. CAG-repeat length of disease alleles can vary from 45 to 87 repeats (Matos et al., 2011). In this study, dermal fibroblasts (named H241) were obtained from a 17-year-old man with spinocerebellar ataxia type 3 (SCA3) with 83 CAG-repeats in the mutant *ATXN3* allele. The fibroblasts were reprogrammed into iPSCs by electroporation with three episomal plasmids encoding human *L-MYC* and *LIN28*, *SOX2* and *KLF4*, and *OCT4* combined with a short hairpin RNA for *P53* (*shP53*). The iPSC line described in this publication was named SCA3.A11. An additional clone from the same patient termed SCA3.A8 was isolated and characterized (data not shown). SCA3.A11 had a structurally and numerically normal karyotype (46, XY) (Fig. 1A) and no integration of episomal reprogramming plasmids (Fig. 1B). The expression of the pluripotency genes *OCT4*, *NANOG*, *SOX2* and *LIN28* was upregulated in iPSCs compared to patient fibroblasts (Fig. 1C) and the gene expression levels were comparable to those of a characterized positive control iPSC line (Rasmussen et al., 2014). Accordingly, all SCA3.A11 iPSCs stained positive for the pluripotency markers OCT4, NANOG, TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4 (Fig. 1D), illustrating the purity of the iPSC line. Pluripotency was supported by the capability of SCA3.A11 to differentiate into the three germ layers

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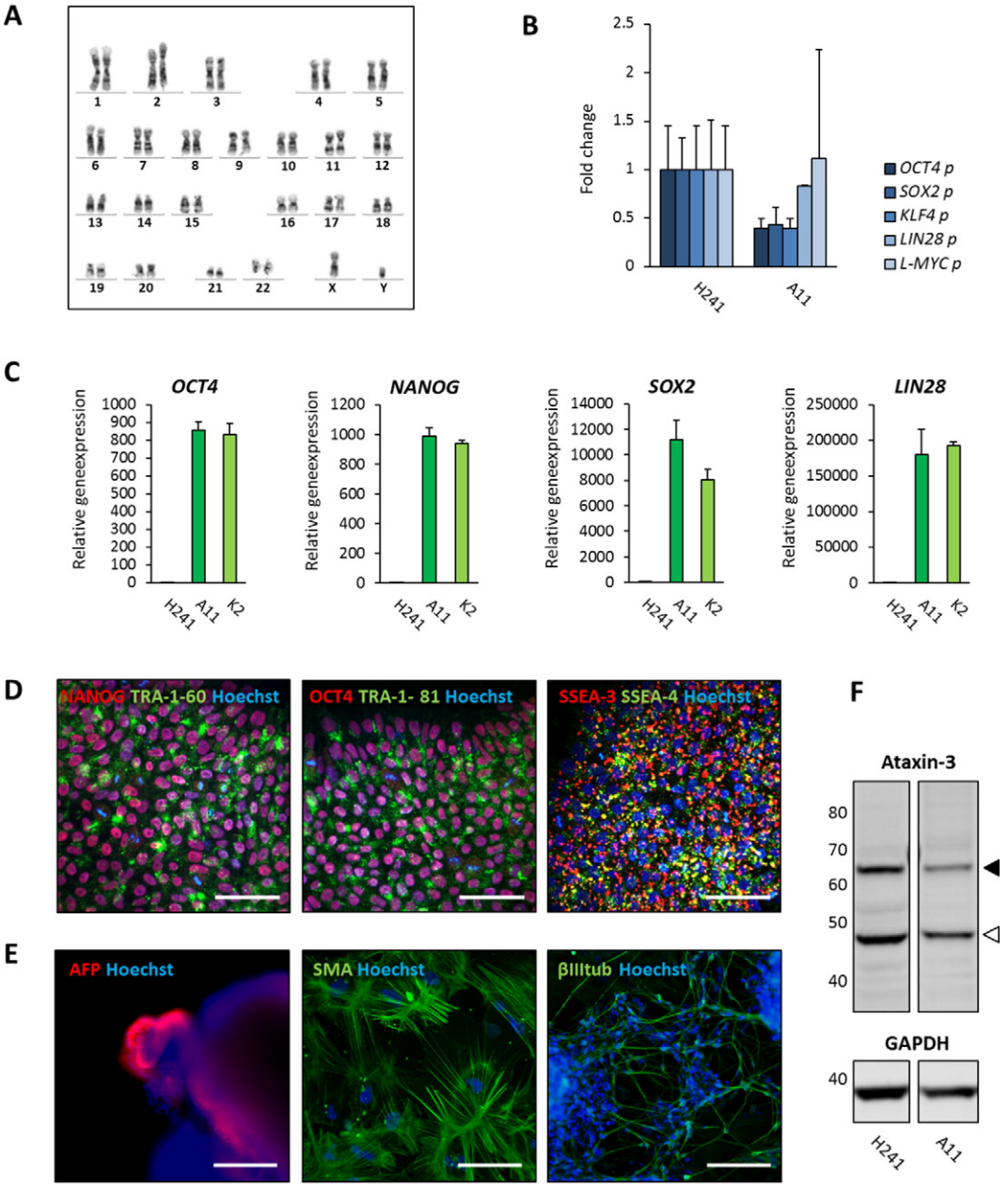

Fig. 1. A. Karyotyping. Representative karyotype of SCA3.A11 iPSCs. B. Integration analysis. Patient fibroblasts (H241) and SCA3.A11 (A11) iPSCs were harvested in duplicates and DNA was isolated for qRT-PCR with reprogramming plasmid specific primers. C_T-values were normalized to the geometric mean of *Hsp90AB1*, *GUSB* and *RPL13A* and fold change was calculated relative to fibroblasts using the $\Delta\Delta C_T$-method (Mean ± S.D). C. Gene expression of pluripotency markers. Patient fibroblasts, SCA3.A11 iPSCs and a previously characterized iPSCs line K2_shP53 (K2) (Rasmussen et al., 2014) were harvested in triplicates and reverse transcribed to cDNA for qRT-PCR. C_T-values were normalized to the geometric mean of *Hsp90AB1*, *GUSB* and *RPL13A* and expression was calculated relative to fibroblasts using the $\Delta\Delta C_T$-method (Mean ± S.D). D. Protein expression of pluripotency markers. Fluorescent immunocytochemistry for pluripotency markers was performed on SCA3.A11 iPSCs. Cell nuclei were stained with Hoechst. Scalebars: 100 μm. E. *In vitro* differentiation. Fluorescent immunocytochemistry showing expression of the endodermal marker α-fetoprotein (AFP), the mesodermal marker smooth muscle actin (SMA) and the ectodermal marker β-III-tubulin (βIIItub) in plated iPSC-derived embryoid bodies. Scalebars: 100 μm. F. Expression of ataxin-3 disease mutation. Western blot showing the protein expression of ataxin-3 in patient fibroblasts and SCA3.A11. White arrowheads show wild type ataxin-3, while black arrowheads indicate the expanded form of ataxin-3. GAPDH was used as a loading control.

in vitro, as confirmed by fluorescent immunocytochemistry showing expression of the endodermal marker α-fetoprotein (AFP), the mesodermal marker smooth muscle actin (SMA) and the ectodermal marker β-III-tubulin (βIIItub) (Fig. 1E). The identity of SCA3.A11 was confirmed by verifying expression of both normal and expanded ataxin-3 proteins by western blot (Fig. 1F). Additionally, the CAG-repeat lengths of the two ataxin-3 alleles were determined to be 23 and 83 repeats in patient fibroblasts and iPSCs by fragment length analysis (data not shown).

Materials and methods

Reprogramming of fibroblasts to iPSCs

Written informed consent was obtained from the SCA3 patient and the study was approved by the regional scientific ethical committee in the Capital Region of Denmark (H-4-2011-157). A skin biopsy was taken from the forearm of a 17-year-old male SCA3 patient, dissected and left in fibroblast medium consisting of Dulbecco's modified eagle's

medium (DMEM) high glucose (Lonza) with 10% fetal bovine serum (FBS) (Biological Industries), 2 mM L-glutamine (Lonza) and 1% penicillin–streptomycin (ThermoFisher Scientific) for 10 days at 37 °C and 5% CO_2 to allow fibroblasts to grow out. Prior to reprogramming, fibroblasts were expanded in fibroblast medium supplemented with 2 ng/ml FGF2 (Sigma-Aldrich). The reprogramming protocol was adopted from a method published byRasmussen et al. (2014). Briefly, 1×10^5 fibroblasts were electroporated with 1 μg 1:1:1 mix of the episomal plasmids pCXLE-hUL (*L-MYC*, *LIN28*, Adgene #27080), pCXLE-hSK (*SOX2*, *KLF4*, Adgene #27078) and pCXLE-hOCT4-shp53-F (*OCT4*, *shP53*, Adgene #27077) (Okita et al., 2011) using the Neon transfection system (Invitrogen) with 2 × 20 ms pulses of 1200 V. The electroporated cells were plated in fibroblast medium without penicillin–streptomycin. From day 1–8 the medium was supplemented with 1% penicillin–streptomycin. On day 7 the cells were split 1:2 with trypsin-EDTA (Sigma-Aldrich) and plated on hESC-qualified matrigel (BD Biosciences). From day 8 the cells were cultured in mTeSR1 (Stem Cell Technologies) supplemented with 0.1% penicillin–streptomycin at 37 °C in 5% CO_2, 5% O_2 and 90% N_2. On day 23–31 single iPSC colonies were manually cut and passaged for expansion. From passage 3 the iPSCs were split every 4–7 days with dispase (ThermoFisher Scientific). The iPSCs were frozen in mFreSR1 (Stem Cell Technologies) with 10 μM rock inhibitor (Sigma-Aldrich). The control iPSC cell line K2_shp53 derived from a healthy donor was previously characterized by Rasmussen et al. (2014) and banked in the European Bank of induced Pluripotent Stem Cells (EBiSC) as BIONi010-B.

Karyotyping

SCA3.A11 iPSCs were treated for 1 h with KaryoMAX colcemid (ThermoFisher Scientific) and harvested in fixative containing 25% acetic acid and 75% methanol. Karyotyping was performed on G-banded metaphase chromosomes using standard procedures. At least 10 metaphases were examined per sample with an approximate resolution of 400–450 bands per haploid genome.

Integration analysis

DNA was isolated using the DNeasy blood & tissue kit (Qiagen) according to manufacturer's protocol. The three episomal plasmids (pCXLE-hUL, pCXLE-hSK and pCXLE-hOCT4-shp53-F) were run as positive controls to verify function of the plasmid specific primers (data not shown). qRT-PCR reactions were run with 2× diluted SsoFast EvaGreen Supermix (Bio-Rad). C_T-values were normalized to the geometric mean of the housekeeping genes *Hsp90AB1*, *GUSB* and *RPL13A* using the $\Delta\Delta C_T$-method. The plasmid specific primers are listed in Table 1.

Quantitative real time polymerase chain reaction (qRT-PCR)

RNA isolation was performed using the RNAeasy mini kit (Qiagen) according to manufacturer's instructions. 0.5 μg RNA/reaction was reverse transcribed using TaqMan reverse transcription reagents (Applied Biosystems). qRT-PCR reactions were run with SsoFast EvaGreen Supermix (Bio-Rad) diluted 2×. C_T-values were normalized to the geometric mean of the three housekeeping genes *Hsp90AB1*, *GUSB* and *RPL13A* using the $\Delta\Delta C_T$-method. Primers are listed in Table 1.

In vitro differentiation

The iPSCs were detached in large clumps with dispase and transferred to ultra-low adherent plates (Stem Cell Technologies) in mTeSR1 with 10 μM rock inhibitor. After 2 days of culture the medium was changed to embryoid body (EB) medium consisting of DMEMF12 (Sigma-Aldrich) supplemented with 20% knockout serum replacement (ThermoFisher Scientific), 1× non-essential amino acids (ThermoFisher Scientific), 2 mM glutamax (ThermoFisher Scientific), 0.1 mM 2-mercaptoethanol (ThermoFisher Scientific) and 1% penicillin–streptomycin. After 7 days EBs were plated on 0.1% gelatin (Sigma-Aldrich) and cultured for 2 weeks in differentiation medium containing DMEM F12 supplemented with 10% FBS, 2 mM glutamax and 1% penicillin–streptomycin.

Table 1
qRT-PCR primers.

	Target	Forward/Reverse primer (5′–3′)
Episomal plasmids	*KLF4* Plasmid	CCACCTCGCCTTACACATGAAGA
		TAGCGTAAAAGGAGCAACATAG
	LIN28 Plasmid	AGCCATATGGTAGCCTCATGTCCGC
		TAGCGTAAAAGGAGCAACATAG
	L-MYC Plasmid	GGCTGAGAAGAGGATGGCTAC
		TTTGTTTGACAGGAGCGACAAT
	OCT4 Plasmid	CATTCAAACTGAGGTAAGGG
		TAGCGTAAAAGGAGCAACATAG
	SOX2 Plasmid	TTCACATGTCCCAGCACTACCAGA
		TTTGTTTGACAGGAGCGACAAT
Pluripotency markers	*LIN28*	AGCCATATGGTAGCCTCATGTCCGC
		TCAATTCTGTGCCTCCGGGAGCAGGGTAGG
	NANOG	TTGGGACTGGTGGAAGAATC
		GATTTGTGGGCCTGAAGAAA
	OCT4	CCCCAGGGCCCCATTTTGGTACC
		ACCTCAGTTTGAATGCATGGGAGAGC
	SOX2	TTCACATGTCCCAGCACTACCAGA
		TCACATGTGTGAGAGGGGCAGTGTGC
House-keeping genes	*GUSB*	TCCGCGCCGACTTCTCTGACA
		AAATGCCGCAGACGCCAGTCC
	HSP90AB1	TCCGGCGCAGTGTTGGGAC
		TCCATGGTGCACTTCCTCAGGC
	RPL13A	TTCCAAGCGGCTGCCGAAGA
		TTCCGGCCCAGCAGTACCTGT

Fluorescent immunocytochemistry

iPSCs and plated EBs were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 20 min and washed in PBS. Subsequently, they were permeabilized with 0.1% triton X-100 (Sigma-Aldrich) for 15 min, blocked with 5% normal donkey serum (Millipore, S30) and 1% BSA (Roche) for 30 min and stained by standard immunofluorescence procedures. Primary antibodies are listed in Table 2. The following secondary antibodies were diluted 1:200: Alexa 594 donkey-anti-rat, Alexa 488 donkey-anti-mouse, Alexa 594 donkey-anti-rabbit and Alexa 594 donkey-anti-goat (all from ThermoFisher Scientific). In addition, cell nuclei were stained with 0.7 μg/ml Hoechst. Images of plated EBs were taken with an Evos fl fluorescent microscope. Images of iPSCs were captured with a Nikon Eclipse Ti 4.10 microscope, Yokogawa CSU-X1 spinning disk confocal scanner. In parallel with analyses of SCA3.A11 iPSCs, patient fibroblasts (H241) were stained as negative controls and the characterized iPSC line K2_shP53 was used as a positive control (data not shown).

Table 2
Primary antibodies.

Marker of	Antigen	Specie	Dilution	Company, catnr.
Pluripotency	NANOG	Rabbit	1:500	Perprotech, 500-P236
	OCT4	Goat	1:200	Santa cruz, sc8628
	SSEA-3	Rat	1:200	Biolegend, MC-631
	SSEA-4	Mouse	1:200	Biolegend, MC813-70
	TRA-1-60	Mouse	1:200	Biolegend, 330602
	TRA-1-81	Mouse	1:200	Biolegend, 330702
Mesoderm	SMA	Mouse	1:500	Dako, M0851
Endoderm	AFP	Rabbit	1:500	Dako, A0008
Ectoderm	βIIItub	Mouse	1:4000	Sigma-Aldrich, T8660
Other	Ataxin-3	Mouse	1:1000	Millipore, MAB5360
	GAPDH	Rabbit	1:1000	Abcam, Ab9485

Western blot

Cells were lysed in Ripa buffer (Sigma-Aldrich) supplemented with 1 complete protease inhibitor cocktail tablet (Roche)/40 ml for 10 min and spun at 15,000 × g for 10 min. 100 mM DTT (Sigma-Aldrich) and NuPAGE LDS Sample Buffer (ThermoFisher Scientific) corresponding to one fourth of the total sample volume were added to the samples. Samples were boiled and run on NuPAGE Novex 4–12% Bis-Tris precast SDS-PAGE gels (ThermoFisher Scientific) and subsequently blotted onto Immobilin-FL PVDF membranes (Merck Millipore). The membranes were blocked in odyssey blocking buffer (LI-COR Biosciences) and incubated with primary antibody ON at 4 °C (Table 2). After washing in PBS/0.1% tween-20 (Merck Millipore) the membranes were incubated with secondary antibodies Alexa Fluor 680 goat-anti-rabbit IgG 1:20,000 (A21077) and Alexa Flour 800 goat-anti-mouse 1:10,000 (926-32210) (both from ThermoFisher Scientific) for 1 h at RT. Emitted light was detected with an infrared Odyssey CLx scanner (LI-COR Odyssey Biosystems).

Fragment length analysis

DNA was isolated with the DNeasy Blood & Tissue Kit (Qiagen) according to manufactures instructions. The CAG-repeat of the *ATXN3* gene was amplified by PCR using the primers 5′-Fam-TGGCCTTTCACA TGGATGTGA-3′ and 5′-CCAGTGACTACTTTGATTCG-3′. 1 μl diluted PCR product, 10 μl formamide (ThermoFisher Scientific) and 0.5 μl SizeStandard LIZ-600 (ThermoFisher Scientific) were mixed and fragment length analysis was performed by capillary electrophoresis on an ABI 3130 Genetic Analyzer. Data were analyzed using the Peak scanner software v1.0 (Applied Biosystems).

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